

UNCLASSIFIED

AD NUMBER
ADB223090
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Nov 96. Other requests shall be referred to Commander, U.S. Army Medical Research and Materiel COMmand, Attn: MCMR-RMI-S, Fort Detrick, Frederick, MD 21702-5012.
AUTHORITY
P. M. Rinehart Deputy Chief of Staff for Info. Mgmt, Army Medical Research and Materiel Cmd, MCMR-RMI-S, Ft. Detrick, MD

THIS PAGE IS UNCLASSIFIED

AD _____

GRANT NUMBER DAMD17-93-J-3033

TITLE: Signalling Pathways of Cooperative Oncogenes and Their Effects on the Transcriptional Control of Cell Cycle Genes in Schwann Cell Transformation (Neurofibromatosis)

PRINCIPAL INVESTIGATOR: Hartmut Land, Ph.D.

CONTRACTING ORGANIZATION: Imperial Cancer Research Fund
London WC2A 3PX England

REPORT DATE: October 1996

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Nov 96). Other requests for this document shall be referred to Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, MD 21702-5012.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

19970502 226

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1996	3. REPORT TYPE AND DATES COVERED Final (22 Sep 93 - 21 Sep 96)	
4. TITLE AND SUBTITLE Signalling Pathways of Cooperative Oncogenes and Their Effects on the Transcriptional Control of Cell Cycle Genes in Schwann Cell Transformation (Neurofibromatosis)			5. FUNDING NUMBERS DAMD17-93-J-3033	
6. AUTHOR(S) Hartmut Land, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Imperial Cancer Research Fund London WC2A 3PX England			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only (proprietary information, Nov 96). Other requests for this document shall be referred to Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, Maryland 21702-5012.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) The co-operation between Ras/Raf and SV40 large T (LT) in the transformation of primary rat Schwann cells is a strikingly synergistic process. We have explored the signalling pathways and the molecular mechanisms involved. Either activated Ras or an inducible Raf onco-protein alone, induce a G1-specific cell cycle arrest. This requires prolonged activation of MAP kinase activity followed by the inhibition of cyclin-dependent kinases (cdk) via induction of the cyclin/cdk inhibitory protein, p21 ^{Cip1} . In contrast, when Raf is activated in the presence of LT or dominant-negative (dn)-p53, the cell cycle arrest and p21 ^{Cip1} induction are specifically abolished and the ability of Raf to increase the specific activity of cyclin-dependent kinases is revealed. Thus Raf-dependent G1 arrest and p21 ^{Cip1} induction require p53 function. This indicates that in this scenario p53 plays a key role in signal integration and determines the specificity of the cellular response to the Ras/Raf pathway independent of DNA damage. It appears that this particular feature of p53 together with its involvement in the induction of apoptosis may represent an important element in its role as a tumour suppressor gene in Schwann cells. In addition, we show that super-induction of cyclin A/cdk2 activity is causally involved in the stimulation of Schwann cell proliferation in the absence of mitogens by Raf and LT. This indicates that co-operation between Raf and LT involves at least two levels of regulation: the loss of p21 ^{Cip1} induction and the co-operative activation of cyclin/cdk complexes.				
14. SUBJECT TERMS Neurofibromatosis, RAS, Schwann Cells, CDC2			15. NUMBER OF PAGES 57	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.


\ ____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

: ____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



PI - Signature

28.11.96

Date

Table of contents	
Introduction	5
Initially proposed research goals	7
Results	8
Ras can be replaced by an inducible Raf protein	8
Raf alters Schwann cell morphology and causes G1 arrest	9
Constitutive activation of MAP kinase by Δ Raf-1:ER in Schwann cells	11
Raf arrests Schwann cells prior to induction of cyclin A	12
Raf inhibits cyclin E- and cyclin A -dependent kinase activity	13
Raf induces p21 ^{Cip1} expression	14
Raf induction of p21 ^{Cip1} and cell cycle arrest are p53 dependent	16
Raf and LT co-operate to increase cyclin A/cdk2 activity and induce DNA synthesis	18
In vivo model	20
Discussion	20
Co-operating oncogenes target cyclin/cdk inhibitors	20
DNA damage-independent role of p53	21
Raf induces p21 ^{Cip1} by a p53-dependent mechanism	22
Raf activates cyclin/cdk activity in presence of a co-operating partner	24
Implications for Neurofibromatosis	25
Methods	26
Conclusions	30
References	32
Appendices	41
Personnel involved	41
Bibliography	41
Figure legends	42
Figures	47

Introduction

Neurofibromatosis is a multi-faceted disease involving the hyperproliferation and malignancy of Schwann cells and fibroblastic cells. In our laboratory a model system to study mechanisms that control rat Schwann cell proliferation and transformation has been established (Ridley et al., 1988; 1989). This system has been used to investigate the role of ras-controlled signalling and the cooperation between ras and nuclear oncogenes, such as SV40 large T (LT), for cell division and tumour progression. This approach appears of particular relevance to the understanding of neurofibromatosis for two reasons:

1) Genetic lesions responsible for the autosomal dominant disorder 'von Recklinghausen neurofibromatosis', have been shown to lead to the decreased expression of a gene named NFI (Viskochil et al., 1990; Cawthon et al., 1990; Wallace et al., 1990; DeClue et al., 1992; Basu et al., 1992). NFI codes for a protein, neurofibromin, which has a catalytic domain with homology to GAP, a protein which activates the intrinsic GTPase of ras proteins. The GAP-related domain (GRD) of neurofibromin has also been shown to stimulate the p21^{ras} GTPase activity (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990). Inhibition of the Ras pathway in malignant schwannomas from patients with neurofibromatosis type 1, using either neutralising antibodies to Ras or by increasing Ras-GAP activity, results in a reversion of the tumour cells, confirming the role of Ras in tumour formation in this disease (Basu et al., 1992; DeClue et al., 1992).

2) Neurofibromatosis patients frequently develop multiple benign neurofibromas, which are composed mainly of Schwann cells. They also have an increased risk of developing malignant tumours derived from Schwann cells or other neural crest derived cell-types. The development of these lesions is likely to involve the accumulation of multiple genetic events. Indeed, in addition to the loss of NF1, malignant neurofibrosarcomas were found to be associated with deletions and mutations of the tumour suppressor gene p53 (Menon et al., 1990). The loss of this tumour suppressor is mimicked by LT, as one of its functions appears to be the inhibition of p53 activity via stable direct interaction (Gannon et al., 1990). In addition, transgenic animals in which LT expression is directed to Schwann cells develop neurocristopathies which resemble Neurofibromatosis type 1, demonstrating that LT expression can also contribute to the development of tumours in this cell type *in vivo* (Mazarakis et al., 1996).

The accumulation of multiple genetic lesions including losses of tumour suppressor gene function and oncogene activation can be mimicked in our *in vitro* Schwann cell transformation system. Moreover, our studies indicate that the elucidation of links between ras signalling and the mechanisms controlling cell cycle progression appears of central importance to the understanding of Schwann cell transformation. The co-operation of Ras and LT in the transformation of primary Schwann cells is a synergistic process in which the cellular response to Ras is dependent on the presence of LT (Ridley et al., 1988). Introduction of activated Ras alone, results in morphologically transformed cells that are growth arrested and only when co-expressed with LT does Ras induce the formation of highly proliferative, anchorage-

independent cultures. In contrast, LT alone lowers the growth factor requirement of cells which otherwise exhibit normal behaviour.

Schwann cells from NF-/- embryos have elevated levels of Ras-GTP, resemble Ras-infected Schwann cells and grow much more slowly than the wild-type cells (Kim, et al., 1995), demonstrating that the observable NF1-/- phenotype is mimicked by activation of the Ras/Raf pathway. The poor growth properties of primary Schwann cells in which the Ras pathway is activated, suggest that further genetic events are required for tumour formation in this cell-type.

Taken together, the convergence and integration of oncogene and/or suppressor gene-controlled signalling pathways in particular at the level of cell cycle regulators (see below) appears essential to the process of tumour progression or oncogene cooperation in Schwann cells. Detailed analysis of these mechanisms should lead to a greater understanding of how Ras-signalling and nuclear oncogenes cooperate to abrogate the growth control of Schwann cells. In the long term this work should provide a basis for the development of strategies and reagents to specifically interfere with the genetic events and the transforming activities that cause Neurofibromatosis and related diseases.

Initially proposed research goals

- 1) Identification and characterisation of the signalling pathways activated by ras.
- 2) Testing whether the expression of proteins involved in the control of the cell cycle can alleviate the ras induced G1 block of the cell cycle.

- 3) Genetically and biochemically characterising the cdc 2 and cyclin A promoter response elements and their corresponding DNA binding proteins which mediate the effects of ras and nuclear oncogenes.
- 4) Development an *in vivo* model to study the tumour forming capabilities of the Schwann cell lines.

Goals 1 and 2 have been intensively pursued. We have made significant progress in these areas during the granting period. The results obtained are detailed and discussed below.

The funding for goal 3 was cut by the site visiting comitte in May 1995. Progress in this area up until then was reported previously and is not included in this final report.

Work towards goal 4 has been fraught with technical difficulties such that it became impossible to design informative animal experiments. It was therefore necessary to abandon this part of the work. The reasons for this are discussed below.

Results

Ras can be replaced by an inducible Raf protein

In order to explore the molecular basis for the co-operation between Ras and LT we have made use of an inducible Raf protein in which an activated Raf kinase has been fused to the oestrogen receptor hormone binding domain (Δ Raf-1:ER) (Samuels et al., 1993). Raf has been shown to act directly downstream of Ras (Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993) and elicits a similar phenotype in some cell types; for example in PC12 cells both activated Ras and Raf proteins are capable of inducing neurite outgrowth (Wood et al., 1993). In primary Schwann cells we

show that the effects of Raf are indistinguishable from those of Ras (see below).

Raf alters Schwann cell morphology and causes G1 arrest

The Raf/oestrogen receptor fusion protein (Δ RafER-1:ER) exhibits kinase and transforming activity in a strictly hormone-dependent fashion (Samuels, et al., 1993). Low passage normal rat Schwann cells were infected with the retroviral vector LXS_N (Miller and Rosman, 1989) or its derivative LXS_N- Δ Raf-1:ER. Several hundred G418 resistant colonies infected with the empty vector (NSE) or the vector encoding the inducible Raf protein (NS Δ RafER) were pooled and expanded. The growth properties of these cells were maintained over several months, in that the cells did not appear to enter crisis or show any significant changes in their ability to quiesce or respond to mitogenic signals. Early passage NS Δ RafER cells were then infected with a second retroviral vector, Babe-Puro (Morgenstern and Land, 1990) or with the Babe-Puro vector expressing SV40 LT. Puromycin resistant colonies expressing both Δ Raf-1:ER and LT (NS Δ RafERLT) or Δ Raf-1:ER and the empty vector (NS Δ RafERBp) were pooled and expanded.

The addition of 4-hydroxy-tamoxifen (TMX) to the NS Δ RafER cells resulted in a dramatic change in cell morphology (Fig 1A). When viewed by time-lapse video microscopy, elongation of the normally flat cells could be seen as early as six hours. In addition to the cells exhibiting a highly refractile phenotype, they also extended processes and became more motile. This motility was not inhibited by cell-cell contact as the cells would move across each other. These morphological changes were indistinguishable from those seen when Schwann cells are injected with Ras protein (Ridley, et al., 1988) or those reported in

Schwann cells isolated from late-stage embryos with a homozygous deletion of the NF1 gene (Kim et al., 1995). The activation of Raf in the cells co-expressing LT (NS Δ RafERLT) led to similar morphological changes and increased cell motility while the addition of TMX to Schwann cells expressing the empty LXS vector (NSE) had no detectable effects on the morphology of the cells (data not shown).

In order to address the effects of the activation of Δ Raf-1:ER on the proliferation of normal Schwann cells, the cells were analysed for BrdU incorporation and DNA content by flow cytometry. The addition of TMX to the NS Δ RafER cells resulted in a cell-cycle arrest in the G1 phase of the cell-cycle, whereas TMX had no effect on the cell-cycle profile of the control NSE cells (Fig1B). This inhibition of cell growth was confirmed by 3 H-thymidine uptake assays, which showed an approximately 80% decrease in DNA synthesis (Figure 1C, right hand panel). When the cells were followed by time lapse microscopy a complete cessation of cell division was observed within 30 hours. This was not associated with any observable cell-death during the 72 hours of the experiment (data not shown). In contrast, the cells co-expressing LT (NS Δ RafERLT) were not inhibited in response to the activation of Δ Raf-1:ER; indeed Δ Raf-1:ER increased the rate of DNA synthesis (Fig. 1C, right hand panel). The mitogenic effect of Raf in the NS Δ RafERLT cells was seen more clearly when the cells were incubated in DMEM containing 2% FCS without GGF or forskolin, conditions in which the NS Δ RafER cells quiesce and the LT cells grow more slowly (Fig. 1C, left hand panel). Under these conditions, addition of TMX to the NS Δ RafERLT cells led to a greater than 3-fold increase in the rate of DNA synthesis as measured by 3 H-thymidine uptake. The activation of Δ Raf-

1:ER had no significant effect on DNA synthesis in the quiescent NS Δ RafER cells (Fig 1C, left hand panel).

Thus Raf behaves similarly to Ras in Schwann cells by inducing distinct morphological changes and a cell-cycle arrest. As with Ras, the cell-cycle block induced by Raf is overcome by co-expressing LT and in this scenario the Raf signal is re-interpreted as a mitogenic stimulus. This would suggest that, in Schwann cells it is the Raf signalling pathway which is responsible for both Ras-induced morphological transformation and the effects of Ras on the cell-cycle.

Constitutive activation of MAP kinase by Δ Raf-1:ER in Schwann cells

As an indicator of the activation of signalling pathways downstream from Raf, we compared the kinetics of MAP kinase activation following mitogen stimulation in the presence and absence of activated Δ Raf-1:ER. Treatment of quiescent NS Δ RafER cells with mitogens resulted in a transient activation of p42-ERK-2, as measured by an in-vitro kinase assay and a phosphorylation-dependent mobility shift (Fig. 1D) which has been shown to be characteristic for MAP kinase activity. In contrast, the addition of TMX to the NS Δ RafER cells in the presence (Fig.1D) or absence (not shown) of exogenous mitogens resulted in the prolonged activation of p42-ERK-2. It therefore appears that the constitutive activity of Δ Raf-1:ER, which results in a cell-cycle arrest, is overriding the growth-promoting signals by the mitogens. The activation of Δ Raf-1:ER in cells co-expressing LT (NS Δ RafERLT), also led to the prolonged activation of p42-ERK-2, with indistinguishable kinetics to those seen in the NS Δ RafER cells (not shown). Thus depending on the presence of LT, a constitutive Raf signal has opposing effects on the cell-cycle.

Raf arrests Schwann cells prior to induction of cyclin A

To investigate the mechanism by which Raf causes a cell cycle arrest and how this is overcome in the NS Δ RafERLT cells, we decided to study the effects of Raf on cyclin/cdk activity. In an initial experiment, we addressed whether the activation of Raf was capable of blocking NS Δ RafER cells from entering the first S phase following growth factor stimulation of quiescent cells, as this would enable us to analyse the effects of Raf in a synchronised cell population.

Subconfluent NS Δ RafER cells were rendered quiescent by incubating them in medium containing 2% FCS for 2 days. Pulse-labelling with 3 H-thymidine showed that entry into S phase occurred 16 to 20 hours following mitogen addition with a peak of DNA synthesis between 20 and 28 hours (Fig. 2A). The co-addition of TMX resulted in an approximately 80% decrease in the proportion of cells reaching the first S phase. This inhibition was not due to a delay in the cells entering S phase since further incubation did not lead to a significant increase in the incorporation of 3 H-thymidine in cells treated with TMX, whereas the untreated cells continued to cycle (not shown).

To determine whether this Raf-induced arrest was linked to changes in cyclin or cdk expression, cell lysates were prepared at various time points and analysed by immunoblotting. Cyclin D1 levels were very low in the quiescent cells (Fig. 2B) and were induced as the cells entered G1. In the presence of TMX, the levels of cyclin D1 were super-induced. Other studies have reported that Ras expression and transformation leads to a marked elevation of cyclin D1 levels (Liu et al., 1995; Winston et al., 1996). Our results demonstrate that the activation of the Raf pathway is sufficient to induce cyclin D1. Cyclin E levels, appeared to be

invariant as the cells progressed from the quiescent state through G1 and were unaffected by the activation of Δ Raf-1:ER, suggesting that modulation of cyclin E levels, is not the principal mechanism controlling cell cycle progression in primary Schwann cells. Cyclin A protein, although present in significant amounts in the quiescent cells, was induced as the cells progressed towards and entered S phase. However, in the cells treated with TMX, this induction of cyclin A was not seen (Fig. 2B). Cdk4 expression increased slightly during G0/G1 progression, while cdk2 levels remained relatively unchanged. However neither cdk4 nor cdk2 levels appeared to be affected by Δ Raf-1:ER activation. These results suggest, that activation of Δ Raf-1:ER leads to a cell cycle arrest in G1 prior to the induction of cyclin A.

Raf inhibits cyclin E- and cyclin A -dependent kinase activity

The finding that the block in G1 appeared to occur prior to the induction of cyclin A was also reflected by the low levels of cyclin A protein observed when growing cells were arrested by activation of Δ Raf-1:ER (Fig 3A, top left hand panel). Immunoprecipitation of cyclin A complexes from these cells showed that there was a corresponding decrease in cyclin A/cdk2 complexes (Fig. 3A top right hand panel) and cyclin A-dependent kinase activity (Fig. 3A, bottom panel). It appears that in Schwann cells, virtually all the cdk2 associated with cyclin A is the faster migrating isoform of cdk2, which has been shown to correspond to the active form phosphorylated on residue Thr160 (Gu et al., 1992). This may reflect high CAK activity in Schwann cells or possibly a preferential association of cyclin A with the activated kinase.

Unlike cyclin A, cyclin E levels were unaffected by activation of Δ Raf-1:ER (Fig. 3B, left hand panel). Moreover, immunoprecipitation of cyclin E complexes from

both growing and arrested NSΔRafER cells showed that there were similar levels of cyclin E/cdk2 complexes in the growing and arrested cells (Fig 3B, upper right hand panel). The cdk2 complexed to cyclin E, as for the cyclin A complexes, was mostly the CAK- phosphorylated form. Interestingly however, the cyclin E-dependent kinase activity associated with these complexes was reduced almost to background levels in the arrested cells (Fig 3B, bottom panel). Thus activation of Raf leads to a dramatic decrease in the specific activity of cyclin E/cdk complexes. The loss of both cyclin E and cyclin A-dependent kinase activities in the arrested cells was reflected by a similar inhibition of cdk2 precipitable H1 kinase activity (data not shown). Cyclin E, cyclin A and cdk2 activity have each been shown to be required for entry into S phase (Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992; Tsai et al., 1993; van den Heuvel and Harlow, 1993; Ohtsubo et al., 1995). Therefore it is likely that the inhibition of these cyclin/cdk complexes is sufficient to arrest the cells in G1.

Raf induces p21^{Cip1} expression

Since cyclin E/cdk2 activity was reduced without a concomitant decrease in the expression of the proteins, we investigated whether Raf activation affected the expression of cdk inhibitors, such as p21^{Cip1} (el-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993; Noda et al., 1994) and p27^{Kip1} (Polyak et al., 1994; Toyoshima and Hunter, 1994). Lysates were prepared from NSΔRafER cells grown in the presence or absence of TMX and the inhibitor levels were analysed by western blotting. Activation of Raf had no effect on p27^{Kip1} levels (Fig. 4A). However, Raf activation induced a large increase in the levels of p21^{Cip1} (Fig. 4B). The addition of TMX to the control NSE cells had no effect on the levels of p21^{Cip1} (data not shown). Samples of each lysate were also

immunoprecipitated with cyclin E antibodies and the precipitated proteins were immunoblotted with p21^{Cip1} antibodies. Figure 4C shows that the induction of Raf in the NSΔRafER cells resulted in a corresponding increase in the amount of p21^{Cip1} associated with cyclin E complexes. Moreover, the induction of p21^{Cip1} as well as the activation of p42-ERK-2 require MEK activity, as both events are sensitive to the MEK inhibitor PD98059 (Fig. 4 D). This clearly indicates the involvement of the MAP kinase pathway in Raf-dependent p21^{Cip1} induction.

To further address whether the induction of p21^{Cip1} is responsible for the suppression of cyclin E-dependent kinase activity and the inhibition of DNA synthesis in Raf arrested cells, we determined the kinetics of these three events. The rate of the ΔRaf-1:ER-induced inhibition of DNA synthesis was analysed by measuring the incorporation of ³H-thymidine in NSΔRafER cells pulse-labelled at various time points. These experiments showed that the growth inhibition induced by ΔRaf-1:ER commenced after a 12 hour lag period with significant decreases in ³H-thymidine uptake apparent after 14 hours and complete inhibition after 24 hours (Fig. 5A). The late onset of the growth arrest induced by ΔRaf-1:ER was paralleled by a similar delay in the induction of p21^{Cip1}, with a small increase seen at 10 hours and maximal induction at approximately 22 hours (Fig. 5B). This delayed appearance of p21^{Cip1} was in contrast to the rapid induction of cyclin D1, which could be seen as early as 3 hours following TMX addition to growing(not shown) or quiescent cells (Fig. 2B). The decrease in cyclin E-dependent kinase activity coincided with the induction of p21^{Cip1} and preceded the inhibition of DNA synthesis (Fig. 5A), arguing that p21^{Cip1} is responsible for the inhibition of the kinase activity which results in the growth arrest of the cells.

Raf induction of p21^{Cip1} and cell cycle arrest are p53 dependent

Schwann cells co-expressing Δ Raf-1:ER and LT (NS Δ RafERLT), no longer arrest in response to Δ Raf-1:ER activation. We were therefore interested to explore whether Δ Raf-1:ER would induce p21^{Cip1} in these cells. Pools of NS Δ RafERLT cells and control NS Δ RafERBp cells, were grown for 30 hours in the presence or absence of TMX. Lysates prepared from these cells were then subjected to western blot analysis to detect p21^{Cip1}. We found that although Δ Raf-1:ER induced p21^{Cip1} in the control cells, LT co-expression resulted in both a decrease in the basal level of p21^{Cip1} expression and an apparent loss of p21^{Cip1} induction (Fig. 6A).

One of the properties of LT is that it can bind to and sequester p53 (Lane and Crawford, 1980; Maltzman et al., 1981). p53 is known to control the regulation of p21^{Cip1} expression (el-Deiry, et al., 1993; Li et al., 1994; Liu, et al., 1995; Macleod et al., 1995) and in recent experiments it has been shown that embryonic fibroblasts isolated from p21^{-/-} mice and a colon cancer cell line with a homozygous deletion of the p21 gene are significantly or completely deficient in their ability to arrest in G1 in response to p53-dependent growth inhibitory signals (Brugarolas et al., 1995; Deng et al., 1995; Waldman et al., 1995). We were therefore interested as to whether the induction of p21^{Cip1} in response to Raf was p53 dependent and whether inactivation of p53 was capable of abolishing the Δ Raf-1:ER-induced growth arrest. NS Δ RafER cells were infected with a BabePuro retroviral vector constructed to encode a dominant negative mutant (dn) of p53 (p53¹⁷⁵), which has been shown to inhibit the activity of endogenous p53 (Kern et al., 1992). Puromycin-resistant colonies were pooled and expanded. Proliferating NS Δ RafERp53¹⁷⁵ were incubated in the presence or absence of TMX. Lysates were prepared and FACS

analysis was carried out on the cells 30 hours after the addition of hormone. Immunoblot analysis of the lysates showed that as with the LT expressing cells, the basal levels of p21^{Cip1} expression were reduced in the cells expressing dn-p53 and Δ Raf-1:ER activation no longer stimulated a significant induction of p21^{Cip1} (Fig. 6A). Consistent with this finding, equivalent levels of p21^{Cip1} were precipitated by cyclin E antibodies from the same lysates (Fig. 6B). Similar results were obtained with NS Δ RafER cells infected with a retrovirus expressing the carboxy-terminal oligomerisation domain of p53 (data not shown). Thus Δ Raf-1:ER induction of p21^{Cip1} in Schwann cells is p53-dependent.

Cyclin D1 induction by Δ Raf-1:ER, unlike p21^{Cip1}, does not appear to be p53 dependent. Immunoblot analysis of lysates of the NS Δ RafERp53¹⁷⁵ cells showed that Raf stimulated a large induction of cyclin D1, similar to that seen in the parental cells (Fig. 6A). This observation demonstrates that p53 is only required for a specific subset of Raf signals. Cyclin D1 levels are lower in NS Δ RafERLT cells, a finding consistent with other reports which have shown that RB-binding proteins such as LT, down regulate cyclin D1 expression and these cells no longer require cyclin D1 function to cycle (Lukas et al., 1994). Raf activation is still able to stimulate cyclin D1 expression in these cells but only to levels found in the uninduced NS Δ RafER cells (Fig. 6A) indicating that the pathway required for cyclin D1 induction remains active in the LT expressing cells.

Cyclin E-dependent kinase assays performed on aliquots of lysates from NS Δ RafERp53¹⁷⁵ showed that in the presence of dn-p53, Δ Raf no longer inhibited the kinase activity but instead stimulated a 2-fold increase in kinase activity (Fig. 6C). Likewise in the NS Δ RafERLT cells Raf also stimulated an

increase in cyclin E-dependent kinase activity. Thus, in the absence of p21^{Cip1} induction, signals from Raf increase cyclin E-dependent kinase activity.

FACS analysis of the NS Δ RafERp53¹⁷⁵ cells showed that their cycling was unaffected by Δ Raf-1:ER activation (Fig. 6C) demonstrating that the Raf induced growth arrest is dependent on normal p53 function.

Raf and LT co-operate to increase cyclin A/cdk2 activity and induce DNA synthesis

Although Schwann cells expressing dn-p53 do not arrest in response to Δ Raf-1:ER, it is only in LT expressing Schwann cells that Δ Raf-1:ER causes an increase in cell cycling. This indicates that in addition to inhibiting p53 activity, LT has additional effects on the cell-cycle (Fig. 7A). It is unlikely that this involves the Δ Raf-1:ER-induced increase in cyclin E-dependent kinase activity, as similar levels of kinase activity were found in cells expressing dn-p53 or LT (Fig. 6D). In contrast, cyclin A-dependent kinase activity was found to be consistently higher in the LT cells (Fig 7B). Western blot analysis showed that NS Δ RafERLT cells had elevated levels of cyclin A and cdk2 when compared with NS Δ RafER or NS Δ RafERp53¹⁷⁵ cells (Fig. 7B), whereas cyclin E levels were unaffected by LT expression (not shown). Cyclin A and cdk2 levels were unaffected by the removal of mitogens (not shown) or by the activation of Raf (Fig. 7B). These findings are consistent with those reported by Oshima et al., 1993, showing that the expression of LT in primary rat lung epithelial cells resulted in a dramatic increase in the expression of cyclin A and cdc2 proteins.

In order to address whether the elevated levels of cyclin A complexes induced by LT may contribute to the ability of Raf to stimulate DNA synthesis in the

NS Δ RafER cells we infected NS Δ RafERp53¹⁷⁵ cells with a Babe hygro retroviral vector constructed to encode cyclin A. Consistent with a role of cyclin A in the increased proliferative response to Δ Raf-1:ER, polyclonal populations of these cells showed an increased proliferative response to Δ Raf-1:ER, whereas Babe hygro infected control cells behaved as the parental cells (Fig. 7A, right-hand panel).

When we measured cyclin A-dependent kinase activity in NS Δ RafERLT cells we found that in the absence of exogenous mitogens, the complexes were mostly inactive. Activation of Δ Raf-1:ER in these conditions stimulated the cells to enter S phase, as measured by FACS analysis or ³H-thymidine uptake (not shown) and resulted in a 8-20 fold increase in kinase activity (Fig. 7C upper panel). This activation was not associated with a detectable change in the levels of cyclin A/cdk2 complexes (Fig. 7C lower panel) and thus represents an increase in the specific activity of the complexes. In the presence of mitogens the cyclin A-dependent kinase activity was elevated but the activation of Raf resulted in a further 2-5 fold further stimulation of kinase activity (Fig. 7C). Activation of cdk2-dependent kinase activity was stimulated to a similar extent (not shown), indicating the increase in kinase activity was at the level of the cyclin A/cdk2 complexes. Thus Raf signalling and LT function converge not only to regulate cyclin E/cdk2 activity but also co-operate to increase cyclin A/cdk2 activity with LT inducing cyclin A/cdk2 levels and Raf increasing the specific activity of these complexes. Thus co-operation between Raf and LT involves both the loss of a cell cycle inhibitor protein and the synergistic activation of cyclin A complexes.

In vivo model

A prerequisite to carry out meaningful experiments with our cell system in vivo is the regulatable expression of Ras or Raf proteins in a living animal. However insurmountable technical difficulties have prevented us from achieving this goal. Although we have constructed a RafER fusion protein with a mutant ER motif which can only bind and be activated by 4-OH-tamoxifen but not by any other steroid hormone (Littlewood et al., 1995), so far regulation of similar fusion proteins in transgenic mice has not been observed (Gerard Evan and Martin Eilers, personal communication). Moreover, attempts to achieve a regulation of Ras proteins in Schwann cells based on the tetracyclin repressor system, which has been shown to be useful for in vivo studies, have also failed. Although we have observed regulation of Ras expression in NIH 3T3 cells after inserting a ras cDNA into a retrovirus carrying a tetracyclin-regulatable cassette (Paulus et al., 1996), Ras expression was constitutive when this virus was introduced into Schwann cells. Therefore the proposed in vivo studies have not been carried out. Not in the least this decision was made in order unnecessary use of experimental animals.

Discussion

Co-operating oncogenes target cyclin/cdk inhibitors

In Schwann cells co-operating oncogenes regulate cyclin/cdk complexes by distinct inhibitory and activating mechanisms. Such regulation plays a key role in connecting cellular signalling pathways to cell-cycle control and determines the specificity of the cellular response to the Ras/Raf pathway. Activation of an inducible Raf protein alone, results in a G1-specific cell cycle arrest associated with a MEK-dependent induction of p21^{Cip1} and the concomitant inhibition of

cyclin/cdk activity. In the presence of LT or dominant-negative mutants of p53 however, the p21^{Cip1} induction is suppressed, the ability of Raf to increase the specific activity of cyclin-dependent kinases is revealed and the growth arrest abolished. This effect is most evident in cells containing LT, as they express higher levels of cyclin A/cdk2 complexes and in these cells Raf activation is sufficient to stimulate proliferation in the absence of exogenous growth factors.

The suppression of Cdk inhibitor proteins may be a common property of immortalizing oncogenes. E1A rescues TGF β cell-cycle arrest by binding to and blocking the inhibitory effects of p27^{Kip1} (Mai et al., 1996). Similarly Myc can overcome cell cycle inhibition by p27^{Kip1} and p16^{INK4a} in fibroblasts via a yet unknown mechanism (B. Amati, personal communication and S. Shellard & H.L., unpublished observations). In addition, fibroblasts isolated from mice carrying a targeted deletion of the INK4a tumour suppressor locus, which encodes the cdk-4/ cdk-6 inhibitory protein p16^{INK4a} can be transformed by Ras or Raf alone (Serrano et al., 1996). This suggests that similar to Raf in Schwann cells, a reduction in the level of cyclin/cdk inhibitory proteins reveals the transforming potential of Ras in fibroblasts.

DNA damage-independent role of p53

The activity of p53 is pivotal to the switch in Raf from a growth inhibitory to a stimulatory signal. Previous work has demonstrated a critical role of p53 in G1 arrest and apoptosis induced by DNA damage after UV irradiation (Kastan et al., 1992; Kuerbitz et al., 1992). Here we show that the activation of the Raf pathway arrests Schwann cells in a p53-dependent fashion in the absence of DNA damage. Thus, it is tempting to speculate that the ability of p53 to modulate the proliferative response to signals such as Ras/Raf activation may

play an important role in its function as a tumour suppressor gene. It is possible that the constitutive activation of the Raf pathway may mimic a damage signal and therefore be sensed by p53 as inappropriate. However, the expression of dominant-negative p53 mutants reduces the growth factor requirement of Schwann cells in the absence of activated Raf (Fig. 7A and data not shown) which suggests a function for p53 in the regulation of normal proliferative signals.

Raf induces p21^{Cip1} by a p53-dependent mechanism

The Raf-induced G1 arrest is preceded by a loss of cyclin E- and cyclin A-dependent kinase activity. The inhibition of cyclin A-dependent kinase activity appears to result from a corresponding decrease in the levels of cyclin A/cdk2 complexes, reflecting a block prior to the induction of cyclin A expression. In contrast, the suppression of cyclin E-dependent kinase is due to an inhibition of the specific activity of cyclin E/cdk2 complexes and correlates with increased expression and binding of the inhibitor protein p21^{Cip1} to the complex. The arrested state induced by Raf is very different from the quiescent state resulting from the removal of growth factors in terms of the balance of cyclin/cdk complexes and inhibitor levels. In addition to elevated p21^{Cip1} expression, Raf-arrested cells also overexpress cyclin D1, whereas p21^{Cip1} and cyclin D1 are barely detectable in quiescent cells. Moreover, in quiescent cells the amount of cdk2 bound to cyclin E is very low whereas the levels of cyclin E/cdk2 complexes in Raf-arrested cells are similar to those in proliferating cells (A. L. & H. L., unpublished observations).

It has previously been shown that overexpression of p21^{Cip1} leads to a cell cycle arrest in G1 (Harper et al., 1995). Other reports have looked at kinase activity

following stimuli which lead to a G1 arrest associated with the induction of cdk inhibitory proteins. p53-dependent G1 arrest in response to DNA damage (Dulic et al., 1994), which is known to be significantly dependent on p21^{Cip1} or the induction of the G1 inhibitory proteins p21^{Cip1} and p27^{Kip1} following the detachment of cells from the substratum (Fang et al., 1996) each results in a G1 arrest associated with a decrease in both cyclin E and cyclin A -dependent kinase activity. Interestingly in both cases, this involves an inhibition of the specific activity of cyclin E kinases, resulting in a block in G1 prior to the induction of cyclin A. Thus, cyclin E/cdk2 complexes appear to be a target for p21^{Cip1} and p27^{Kip1} when induced by various mechanisms. This is consistent with cyclin E/cdk2 activity being required for the induction of cyclin A expression. It is possible that p21^{Cip1} is also inhibiting cyclin D-dependent kinase activity, although due to the technical difficulties in measuring cyclin D-dependent kinase activity, this was not further investigated.

The regulation of p21^{Cip1} expression appears complex. DNA damage signals are known to result in the induction of p21^{Cip1} in a p53-dependent fashion via two conserved p53 binding sites in the promoter (Dulic, et al., 1994; Michieli et al., 1994; Macleod, et al., 1995). The G1 arrest associated with these signals has been shown to be partly or fully dependent upon p21^{Cip1} (Brugarolas, et al., 1995; Deng, et al., 1995; Waldman, et al., 1995). However, p21^{Cip1} expression is induced as an immediate early gene, by various mitogens and differentiation agents and by the growth inhibitory peptide TGF β , in a p53-independent fashion (Jiang et al., 1994; Li, et al., 1994; Michieli, et al., 1994; Steinman et al., 1994) and the response to mitogens can be blocked or mimicked by inhibitors or activators of the MAP kinase pathway (Liu et al., 1996). The role of the induction of p21^{Cip1} as cells enter the cycle is unclear, although it has been

proposed that p21^{Cip1} may act as an assembly factor of cyclin/cdk complexes (Zhang et al., 1994). Elevated levels of p21^{Cip1} expression are also associated with the differentiated state of specific tissues and have been postulated to be involved in the maintenance of the quiescent differentiated state (Halevy et al., 1995; Macleod et al., 1995). The latter has also been shown to be a p53-independent mechanism. In Schwann cells, we show that the Raf-dependent induction of p21^{Cip1} is a p53-dependent process and thus differs from the immediate early response seen following mitogen stimulation. It will be of interest to dissect the mechanisms involved in this p53-dependent induction.

In contrast to the rapid induction of cyclin D1 in response to Raf activation, the induction of p21^{Cip1} is delayed. This suggests that the mechanism by which Raf stimulates p21^{Cip1} expression may be indirect. The kinetics of the induction however, may explain how activation of the same pathway i.e. the MAP-kinase pathway, can result in opposing effects on the cell cycle. If constitutive activation of the MAP kinase pathway for several hours is required to induce p21^{Cip1}, this may partly explain why transient activation of the same pathway by mitogens, stimulates rather than inhibits the cell cycle.

Raf activates cyclin/cdk activity in presence of a co-operating partner

In the absence of normal p53 function, Raf activation results in an increase in cyclin/cdk activity, presumably by a mechanism which can be suppressed by p21^{Cip1}. This demonstrates that the effects of an oncogene cannot be gauged solely by the introduction of a single oncogene into a primary cell, as the specificity of the cellular response can depend on the expression of other co-operating genes. In addition to inhibiting p53, LT leads to the constitutive over-

expression of cyclin A/cdk2 complexes with Raf activation causing a super-induction of the kinase activity. In this context it is noteworthy that although loss of functional p53 abolishes the Raf-induced cell cycle arrest, only cells expressing LT respond to Raf activation with increased proliferation. The elevation of the cyclin A complexes appears to be at least partly responsible for the ability of Raf to stimulate proliferation in these cells, as p53-defective cells infected with a cyclin A carrying retrovirus are similarly induced to cycle by Raf. Preliminary experiments indicate that the mechanism of this activation does not involve further alterations in the levels of p21^{Cip1} or p27^{Kip1} or cdc25-dependent dephosphorylation of cdk2 (AL & HL unpublished observations). As the activation of cyclin-dependent kinase activity appears to be induced in a similar fashion by growth factors (Fig. 7c) it will be important to identify the mechanisms involved in this process.

Implications for Neurofibromatosis

The model system which we have developed demonstrates how progressive genetic changes contribute to the transformed phenotype and describe some of the molecular mechanisms involved. Raf alone, leads to a change in the morphology of the cells, coincident with an increase in cell motility and the induction of growth factor secretion, all of which can be imagined to disrupt the micro-environment of the cell. However, these cells are growth arrested due to the induction of p21^{Cip1}. Inactivation of p53, abolishes the growth arrest, whilst retaining the other Raf-dependent properties. The co-expression of LT, in addition, results in the co-operative activation of cyclin A- dependent kinases and these cells acquire the ability to proliferate in response to the Raf signal in the absence of exogenous mitogens.

In Neurofibromatosis Type I, the Ras pathway is activated via inactivation of neurofibromin, a Ras-GAP (Ballester, et al., 1990; Martin, et al., 1990; Xu, et al., 1990; Xu, et al., 1990). Inhibition of the Ras pathway in tumour cells derived from these patients, results in a reversion of the tumour cells, confirming the role of Ras in tumour formation in this disease (Basu, et al., 1992; DeClue, et al., 1992). We have shown that activation of Raf in primary Schwann cells, as previously reported for Ras (Ridley, et al., 1988), results in a growth arrest of the cells. In addition, a recent report described the isolation of Schwann cells from NF-/- embryos. These Schwann cells were shown to have elevated levels of Ras-GTP, resembled Ras- infected Schwann cells and grew much more slowly than the wild-type cells (Kim, et al., 1995), demonstrating that the observable NF-/- phenotype is mimicked by activation of the Ras/Raf pathway. The poor growth properties of primary Schwann cells in which the Ras pathway is activated, suggest that further genetic events are required for tumour formation in this cell-type. It will be of great interest to investigate whether the benign and malignant tumours found in patients suffering from Neurofibromatosis type I, have further genetic defects which result in the suppression of the effects of p21^{Cip1}. These may involve the loss of p21^{Cip1} or p53 expression, mutations which affect cyclin/p21^{Cip1} interaction or increases in the levels of cyclin/ cdk complexes. Interestingly, reports of p53 mutations in neurofibrosarcomas from NF1 patients have previously been reported (Menon et al., 1990).

Methods

Cell culture

Schwann cells were purified from 2-3 day old Wistar rats, as previously described (Brockes, et al., 1979; Ridley, et al., 1988). The Schwann cells were

routinely cultured at 37° C (10% CO₂) in Dulbecco's modified Eagles medium (DMEM) with 1.5 mg/ml glucose, supplemented with 3% FCS, 1μM forskolin (Calbiochem) and Glial Growth Factor (GGF) (a kind gift from Mark Noble), on dishes pre-coated with poly-L-lysine (Sigma). Throughout all the experiments phenol red -minus medium and charcoal-stripped serum were used. To render the cells quiescent, the cells were washed twice in DMEM and incubated in either DMEM supplemented with 2%FCS or SATO mix, a mitogen-free supplement (Raff et al., 1983), for 48 hours.

Retroviral vectors

The Xho1/Cla1(blunted) fragment encoding ΔRaf-1:ER (Samuels, et al., 1993) was subcloned into the Xho1/BamH1(blunted) site of the retroviral vector (Miller and Rosman, 1989). The BamH1 fragment encoding SV40LT (Jat et al., 1986) and a BamH1 fragment encoding human p53¹⁷⁵ (provided by David Lane)(Vojtesek et al., 1992) were subcloned into the BamH1 site of the Babe-Puro retroviral (Morgenstern and Land, 1990). A sequenced PCR fragment encoding amino acids 302-390 of murine p53 (p53^{CT}) was subcloned into the BamH1/EcoR1 site of Babe-puro (a kind gift of Trevor Littlewood). A BamH1/Sal1 fragment encoding the human cyclin A gene (a kind gift from Jonathan Pines) was subcloned into the BamH1/Sal1 sites of the Babe Hygro retroviral vector (Morgenstern and Land, 1990). Each of the constructs were transfected, using the standard calcium phosphate method, into the packaging cell line GP+E (Markowitz et al., 1990) and G418, hygromycin or puromycin colonies were pooled and expanded.

Infection of Schwann cells

Schwann cells were infected by co-cultivation, at a 1:2 ratio, with the producer cell lines, which had been pretreated for 2 hours with 20μg/ml mitomycin C

(Sigma). 2-3 days after plating the cultures were transferred into selective medium containing 400µg/ml G418 (Gibco) or 0.4µg/ml puromycin (Sigma) as appropriate. Drug resistant colonies were pooled and expanded.

Facs analysis and DNA synthesis assays

1-2 x 10⁶ cells were preincubated for 4 hours with 10µM BrdU(Sigma), trypsinised and then fixed in 80% ethanol. The fixed cells were then incubated with fluorescein isothiocyanate-cojugated anti BrdU antibodies (Becton-Dickinson) and stained with propidium iodide containing RNase (20 µg/ml). Replicative DNA synthesis and DNA content were analysed using bivariate flow cytometry. For DNA synthesis assays, 5 x10⁴ cells were seeded in triplicate into 6 well dishes in conditions as described in the figure legends. ³H-thymidine was used at a concentration of 0.5µCi/ml. At the indicated times the cells were lysed in 1% SDS and the TCA precipitable material was filtered and counted.

Western Blot Analysis

The cells were lysed in buffer A (1% NP40, 50mM Tris pH. 8, 150mM NaCl, 10µg/ml aprotinin, leupeptin and pepstatin, 20mM NaF, 1mM Na₃VO₄, 100µg/ml PMSF). Protein concentration was determined using the Biorad protein assay. 30 µg of lysate were resolved by SDS-PAGE and electroblotted onto Immobilon P membranes (Millipore). The following antibodies were used :anti-p42 ERK-2(122), provided by Chris Marshall (Leevers and Marshall, 1992); anti-cyclin.D1, provided by Gordon Peters(Bates et al., 1994); anti-cyclin E (Santa-Cruz, sc-481); anti-cyclin A-E-23, provided by Julian Gannon and Tim Hunt; anti cdk-4 (Santa-Cruz, sc-749); anti cdk-2 (Santa Cruz, sc-163); anti-p27 (Santa Cruz, sc-528); anti-p21(CP36), provided by Wade Harper:

Immunoreactive bands were visualised using enhanced chemiluminescence detection (Amersham International PLC).

Immunoprecipitations and kinase assays

Cells were lysed in buffer A. 300µg of lysate were incubated with 10µg of antibody (cyclin E-Santa-Cruz 481, cyclin A-E72, provided by Julian Gannon and Tim Hunt (Slingerland et al., 1994), cdk-2-Santa-Cruz 163) for 1 hour at 37°C, followed by incubation for a further hour with protein A or protein G sepharose, as appropriate. The beads were washed five times in buffer A and either subjected to Western blot analysis or kinase assays. For kinase assays the beads were washed a further two times in kinase buffer (50mM Tris pH7.5, 10mM MgCl₂, 1mM DTT) and then resuspended in 50µl of kinase buffer supplemented with 50µM ATP, 5µCi [³²P]ATP and 10µg of histone H1(Boehringer Mannheim) for 30 minutes at 37°C. The samples were resolved by SDS-PAGE and exposed to Kodak X-OMAT AR. The kinase assays were quantified using ImageQuant by Molecular Dynamics.

Conclusions

We show that in rat Schwann cells Raf and LT co-operate to regulate cyclin/cdk activity and have investigated the molecular mechanisms involved. Activated Ras and Raf cause a G1- specific cell cycle arrest, while together with SV40 large T (LT) or dominant-negative p53 Ras/Raf co-operate to induce rapid proliferation. The G1 arrest induced by Raf alone requires MEK activity and involves prolonged activation of MAP kinase activity followed by the inhibition of cyclin-dependent kinases (cdk) via induction of the cyclin/cdk inhibitory protein, p21^{Cip1}. In parallel, Raf also induces an altered cell morphology and increased cell motility. When Raf is activated in the presence of LT or dominant-negative (dn)-p53, cell cycle arrest and p21^{Cip1} induction are specifically abolished while the other Raf-induced parameters remain unchanged. Thus Raf-dependent G1 arrest and p21^{Cip1} induction require normal p53 function. This indicates that in this scenario p53 plays an important role in the integration of cellular signals as well as in determining the specificity of cellular responses. LT is more potent in co-operation with Raf than dn-p53. In contrast to dn-p53, LT permits Raf to induce Schwann cell proliferation in the absence of exogenous growth factors. A LT-induced large increase in the levels of cyclin A/cdk2 complexes is at least partly responsible for this effect. However, in the absence of exogenous mitogens these complexes are mostly inactive. Activation of Raf in the presence of LT stimulates cyclin A/cdk2 activity which is followed by G1/S transition. This indicates that co-operation between Raf and LT involves both the loss of p21^{Cip1} induction and the co-operative activation of a cyclin/cdk complex (see Fig. 8). Our findings suggest that it will be of great interest to investigate whether the benign and malignant tumours found in patients suffering from Neurofibromatosis type I, have genetic defects which result in the suppression of the effects of p21^{Cip1}. These may not only be p53 mutations (Menon et al.,

1990) but also involve the loss of p21^{Cip1} expression, mutations which affect cyclin/p21^{Cip1} interaction or increases in the levels of cyclin/ cdk complexes.

References

- Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M. and Collins, F. 1990. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell* **63**: 851-859.
- Basu, T.N., Gutmann, D.H., Fletcher, J.A., Glover, T.W., Collins, F.S. and Downward, J. 1992. Aberrant regulation of ras proteins in malignant tumour cells from type 1 neurofibromatosis patients. *Nature* **356**: 713-715.
- Bates, S., Parry, D., Bonetta, L., Vousden, K., Dickson, C. and Peters, G. 1994. Absence of cyclin D/cdk complexes in cells lacking functional retinoblastoma protein. *Oncogene* **9**: 1633-1640.
- Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T. and Hannon, G.J. 1995. Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* **377**: 552-557.
- Cawthon, R.M., Weiss, R., Xu, G.F., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P. and White, R. 1990. A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell* **62**: 193-201.
- DeClue, J.E., Papageorge, A.G., Fletcher, J.A., Diehl, S.R., Ratner, N., Vass, W.C. and Lowy, D.R. 1992. Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. *Cell* **69**: 265-273.
- Deng, C., Zhang, P., Harper, J.W., Elledge, S.J. and Leder, P. 1995. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**: 675-684.
- Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J. and Reed, S.I. 1994. p53-dependent inhibition of cyclin-dependent kinase

- activities in human fibroblasts during radiation-induced G1 arrest. *Cell* **76**: 1013-1023.
- el-Deiry, W., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**: 817-825.
- Fang, F., Orend, G., Watanabe, N., Hunter, T. and Ruoslahti, E. 1996. Dependence of cyclin E-CDK2 kinase activity on cell anchorage. *Science* **271**: 499-502.
- Fanidi, A., Harrington, E.A. and Evan, G.I. 1992. Cooperative interaction between c-myc and bcl-2 proto-oncogenes. *Nature* **359**: 554-556.
- Girard, F., Strausfeld, U., Fernandez, A. and Lamb, N.J. 1991. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* **67**: 1169-1179.
- Gu, Y., Rosenblatt, J. and Morgan, D.O. 1992. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. *Embo J* **11**: 3995-4005.
- Halevy, O., Novitch, B.G., Spicer, D.B., Skapek, S.X., Rhee, J., Hannon, G.J., Beach, D. and Lassar, A.B. 1995. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**: 1018-21.
- Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**: 805-816.
- Harper, J.W., Elledge, S.J., Keyomarsi, K., Dynlacht, B., Tsai, L.H., Zhang, P., Dobrowolski, S., Bai, C., Connell, C.L., Swindell, E. and et, a.I. 1995. Inhibition of cyclin-dependent kinases by p21. *Mol Biol Cell* **6**: 387-400.
- Hunter, T. 1991. Cooperation between oncogenes. *Cell* **64**: 249-270.

- Jat, P.S., Cepko, C.L., Mulligan, R.C. and Sharp, P.A. 1986. Recombinant retroviruses encoding simian virus 40 large T antigen and polyomavirus large and middle T antigens. *Mol Cell Biol* **6**: 1204-1217.
- Jiang, H., Lin, J., Su, Z.Z., Collart, F.R., Huberman, E. and Fisher, P.B. 1994. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. *Oncogene* **9**: 3397-3406.
- Kastan, M.B., Zhan, Q., el, D.W., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B. and Fornace, A.J. 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* **71**: 587-597.
- Kern, S.E., Pieterpol, J.A., Thiagalingam, S., Seymour, A., Kinzler, K.W. and Vogelstein, B. 1992. Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science* **256**: 827-830.
- Kim, H.A., Rosenbaum, T., Marchionni, M.A., Ratner, N. and DeClue, J.E. 1995. Schwann cells from neurofibromin deficient mice exhibit activation of p21ras, inhibition of cell proliferation and morphological changes. *Oncogene* **11**: 325-335.
- Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V. and Kastan, M.B. 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc Natl Acad Sci U S A* **89**: 7491-7495.
- Land, H., Parada, L.F. and Weinberg, R.A. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* **304**: 596-602.
- Lane, D.P. and Crawford, L.V. 1980. The complex between simian virus 40 T antigen and a specific host protein. *Proc R Soc Lond B Biol Sci* **210**: 451-463.

- Leevers, S.J. and Marshall, C.J. 1992. Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoprotein. *Embo J* **11**: 569-574.
- Li, Y., Jenkins, C.W., Nichols, M.A. and Xiong, Y. 1994. Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21. *Oncogene* **9**: 2261-2268.
- Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G. and Evan, G.I. 1995. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res* **23**: 1686-1690.
- Liu, J.J., Chao, J.R., Jiang, M.C., Ng, S.Y., Yen, J.J. and Yang, Y.H. 1995. Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. *Mol Cell Biol* **15**: 3654-3663.
- Lloyd, A.C., Obermüller, F., Staddon, S., Barth, C.F., McMahon, M. and Land, H. 1996. Co-operating oncogenes converge to regulate cyclin/cdk complexes. *Genes Dev.* in revision.
- Lukas, J., Muller, H., Bartkova, J., Spitkovsky, D., Kjerulff, A.A., Jansen, D.P., Strauss, M. and Bartek, J. 1994. DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1. *J Cell Biol* **125**: 625-638.
- Macleod, K.F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B. and Jacks, T. 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev* **9**: 935-944.
- Macleod, K.F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B. and Jacks, T. 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev* **9**: 935-44.

- Mai, A., Poon, R.Y.C., Howe, P.H., Toyoshima, H., Hunter, T. and Harter, M.L. 1996. Inactivation of p27^{Kip1} by the viral E1A oncoprotein in TGFB-treated cells. *Nature* **380**: 262-265.
- Maltzman, W., Oren, M. and Levine, A.J. 1981. The structural relationships between 54,000-molecular-weight cellular tumor antigens detected in viral- and nonviral-transformed cells. *Virology* **112**: 145-56.
- Markowitz, D., Hesdorffer, C., Ward, M., Goff, S. and Bank, A. 1990. Retroviral gene transfer using safe and efficient packaging cell lines. *Ann N Y Acad Sci* **612**: 407-414.
- Martin, G.A., Viskochil, D., Bollag, G., McCabe, P.C., Crosier, W.J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P., Cawthon, R.M. and et, a.l. 1990. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. *Cell* **63**: 843-9.
- Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J. and Kato, J.Y. 1994. D-type cyclin-dependent kinase activity in mammalian cells. *Mol Cell Biol* **14**: 2066-76.
- Mazarakis, N.D., Yannoutsos, N., El-Jabbour, J.N., Hatton, W., Fletcher, R. and Grosveld, F. 1996. Neurocristopathy resembling neurofibromatosis type 1 in an NGF-SV40 transgenic line. *Genes to Cells* **1**: 125-137.
- Menon, A.G., Anderson, K.M., Riccardi, V.M., Chung, R.Y., Whaley, J.M., Yandell, D.W., Farmer, G.E., Freiman, R.N., Lee, J.K., Li, F.P. and et, a.l. 1990. Chromosome 17p deletions and p53 gene mutations associated with the formation of malignant neurofibrosarcomas in von Recklinghausen neurofibromatosis. *Proc Natl Acad Sci U S A* **87**: 5435-9.
- Michieli, P., Chedid, M., Lin, D., Pierce, J.H., Mercer, W.E. and Givol, D. 1994. Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res* **54**: 3391-3395.

- Miller, A.D. and Rosman, G.J. 1989. Improved retroviral vectors for gene transfer and expression. *Biotechniques* **7**: 980-982.
- Moodie, S.A., Willumsen, B.M., Weber, M.J. and Wolfman, A. 1993. Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase [see comments]. *Science* **260**: 1658-1661.
- Morgenstern, J.P. and Land, H. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res* **18**: 3587-3596.
- Noda, A., Ning, Y., Venable, S.F., Pereira, S.O. and Smith, J.R. 1994. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res* **211**: 90-98.
- Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M. and Pagano, M. 1995. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol Cell Biol* **15**: 2612-2624.
- Oshima, J., Steinmann, K.E., Campisi, J. and Schlegel, R. 1993. Modulation of cell growth, p34cdc2 and cyclin A levels by SV-40 large T antigen. *Oncogene* **8**: 2987-2993.
- Pagano, M., Pepperkok, R., Verde, F., Ansorge, W. and Draetta, G. 1992. Cyclin A is required at two points in the human cell cycle. *Embo J* **11**: 961-971.
- Paulus, W., Baur, I., Boyce, F.M., Breakefield, X.O. and Reeves, S.A. 1996. Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. *J Virol* **70**: 62-67.
- Polyak, K., Kato, J.Y., Solomon, M.J., Sherr, C.J., Massague, J., Roberts, J.M. and Koff, A. 1994. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev* **8**: 9-22.

Raff, M.C., Miller, R.H. and Noble, M. 1983. A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium.

Nature **303**: 390-396.

Ridley, A.J., Paterson, H.F., Noble, M. and Land, H. 1988. Ras-mediated cell cycle arrest is altered by nuclear oncogenes to induce Schwann cell transformation.

Embo J **7**: 1635-1645.

Ridley, A.J., Davis, J.B., Stroobant, P. and Land, H. 1989. Transforming growth factors- b1 and b 2 are mitogens for rat Schwann cells. *J Cell Biol* **109**: 4319-3424.

Ruley, H.E. 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature* **304**: 602-606.

Samuels, M.L., Weber, M.J., Bishop, J.M. and McMahon, M. 1993. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. *Mol Cell Biol* **13**: 6241-6252.

Serrano, M., Lee, H.-W., Chin, L., Cordon-Cardo, C., Beach, D. and DePinho, R.A. 1996. Role of the INK4a Locus in Tumor Suppression and Cell Mortality. *Cell* **85**: 27-37.

Slingerland, J.M., Hengst, L., Pan, C.H., Alexander, D., Stampfer, M.R. and Reed, S.I. 1994. A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor beta-arrested epithelial cells. *Mol Cell Biol* **14**: 3683-3694.

Steinman, R.A., Hoffman, B., Iro, A., Guillouf, C., Liebermann, D.A. and el, H.M. 1994. Induction of p21 (WAF-1/CIP1) during differentiation. *Oncogene* **9**: 3389-3396.

Toyoshima, H. and Hunter, T. 1994. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* **78**: 67-74.

- Tsai, L.H., Lees, E., Faha, B., Harlow, E. and Riabowol, K. 1993. The cdk2 kinase is required for the G1-to-S transition in mammalian cells. *Oncogene* **8**: 1593-1602.
- Van Aelst, L., Barr, M., Marcus, S., Polverino, A. and Wigler, M. 1993. Complex formation between RAS and RAF and other protein kinases. *Proc Natl Acad Sci U S A* **90**: 6213-6217.
- van den Heuvel, S. and Harlow, E. 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262**: 2050-2054.
- Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74**: 205-214.
- Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., White, R. and O'Connell, P. 1990. Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. *Cell* **62**: 187-192.
- Vojtesek, B., Bartek, J., Midgley, C.A. and Lane, D.P. 1992. An immunochemical analysis of the human nuclear phosphoprotein p53. New monoclonal antibodies and epitope mapping using recombinant p53. *J Immunol Methods* **151**: 237-244.
- Wallace, M.R., Marchuk, D.A., Andersen, L.B., Letcher, R., Odeh, H.M., Saulino, A.M., Fountain, J.W., Brereton, A., Nicholson, J., Mitchell, A.L., Brownstein, B.H. and Collins, F.S. 1990. Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. *Science* **249**: 181-186.
- Waldman, T., Kinzler, K.W. and Vogelstein, B. 1995. p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res* **55**: 5187-5190.
- Warne, P.H., Viciani, P.R. and Downward, J. 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. *Nature* **364**: 352-355.

- Winston, J.T., Coats, S.R., Wang, Y.Z. and Pledger, W.J. 1996. Regulation of the cell cycle machinery by oncogenic ras. *Oncogene* **12**: 127-134.
- Wood, K.W., Qi, H., D'Arcangelo, G., Armstrong, R.C., Roberts, T.M. and Halegoua, S. 1993. The cytoplasmic raf oncogene induces a neuronal phenotype in PC12 cells: a potential role for cellular raf kinases in neuronal growth factor signal transduction. *Proc Natl Acad Sci U S A* **90**: 5016-5020.
- Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. 1993. p21 is a universal inhibitor of cyclin kinases [see comments]. *Nature* **366**: 701-704.
- Xu, G.F., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R. and Tamanoi, F. 1990. The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of *S. cerevisiae*. *Cell* **63**: 835-41.
- Xu, G.F., O'Connell, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R. and et, a.l. 1990. The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell* **62**: 599-608.
- Zhang, H., Hannon, G.J. and Beach, D. 1994. p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev* **8**: 1750-1758.
- Zindy, F., Lamas, E., Chenivesse, X., Sobczak, J., Wang, J., Fesquet, D., Henglein, B. and Brechot, C. 1992. Cyclin A is required in S phase in normal epithelial cells. *Biochem Biophys Res Commun* **182**: 1144-1154.

Appendices

Personnel involved:

Hartmut Land Ph D, principal investigator

Alison Lloyd Ph D, post-doctoral fellow

Frank Obermüller, graduate student

Susan Staddon B.A. technician

Bibliography

Lloyd, A.C., Obermüller, F., Staddon, S., Barth, C.F., McMahon, M. and Land, H.
1996. Co-operating oncogenes converge to regulate cyclin/cdk complexes.
Genes Dev. in revision.

Meeting abstracts

Signalling by co-operating oncogenes converges on cyclin/cdk regulation.

Land, H, Lloyd, A.C. and Obermüller, F.

EMBL Conference on 'Oncogenes and Growth Control', Heidelberg, April, 1996

Co-operating oncogenes target cyclin/cdk activity

Lloyd, A.C., Obermüller, F. and Land, H.

EMBL Conference on 'Oncogenes and Growth Control', Heidelberg, April, 1996

Oncogene co-operation in Schwann cells targets cyclin/cdk activity

Lloyd, A.C., Obermüller, F. and Land, H.

FASEB Conference on Neurofibromatosis, Snowmass Co., July, 1996

Figure legends

Figure 1. Activation of Raf in primary Schwann cells leads to the constitutive activation of MAP kinase, morphological alterations and a G1 specific cell-cycle arrest.

(A) Phase-contrast micrographs of pools of primary Schwann cells infected with a retrovirus encoding Δ Raf-1:ER, grown in the presence or absence of 200nM 4-OH-tamoxifen (TMX) for 30 hours.

(B) Pools of asynchronously growing NS Δ RafER cells and control NSE cells were cultured in the presence or absence of 200nM TMX for 30 hours. Cells were trypsinised and examined for DNA content by propidium iodide staining and flow cytometry and also analysed for BrdU uptake in the four hours prior to lysis (see insets).

(C) NSE, NS Δ RafER and NS Δ RafERLT were seeded into DMEM supplemented with 2% FCS. 48 hours later the cells were treated with TMX or ethanol (- TMX) in the absence (left hand panel) or presence (right hand panel) of fresh medium supplemented with 3% FCS, forskolin and GGF. 3 H-thymidine was added 12 hours later and the cells were lysed after a further 16 hours. TCA precipitable material was filtered and counted.

(D) NS Δ RafER cells were quiesced for 48 hours in DMEM supplemented with 2% FCS. The cells were lysed at the indicated time points following the addition of fresh medium supplemented with GGF and forskolin and 200nM TMX. The lysates were equalised for protein content and then western blotted using the anti-ERK-2 polyclonal antiserum 122.

Figure 2. Raf arrests Schwann cells in G1 prior to the induction of cyclin A.

(A) NSΔRafER cells were quiesced for 48 hours in DMEM supplemented with 2% FCS. The cells were stimulated with fresh medium supplemented with forskolin and GGF in the presence or absence of 200nM TMX. The cells were pulse-labelled with ^3H -thymidine at four hourly intervals and TCA precipitable material was filtered and counted in a scintillation counter.

(B) Protein lysates were prepared at the time points indicated and were analysed by western blotting with antibodies specific for the indicated proteins.

Figure 3. Raf activation inhibits cyclin A- and cyclin E-dependent kinase activity. Asynchronously growing NSΔRafER cells were cultured in the presence (+) or absence (-) of 200nM TMX for 30 hours. Protein lysates were prepared and standardised for protein content. The left hand upper panels show western analysis for cyclin A (A) and cyclin E (B).

300μg of the lysates were subjected to immunoprecipitation with a cyclin A MAb (A) or a cyclin E antibody (B). The controls were protein G-sepharose beads alone, for the cyclin A antibody or a peptide block for the cyclin E antibody. The immunoprecipitates were either western blotted with a cdk2 antibody (upper right hand panels) or assayed for histone H1 kinase activity (lower panels). * Alternative spliced form of cdk2 seen in rodent cells (Matsushime et al., 1994).

Figure 4. Activation of Raf leads to an increase in p21^{Cip1}.

NSΔRafER cells were grown for 30 hours in the presence of TMX(+) or the solvent control (-). 30μg of protein lysates were subjected to immunoblot analysis with an anti-p27 antibody (A) or an anti-p21 antibody (B).

(C) 300μg of the lysates were immunoprecipitated with the cyclin E antibody and western blotted with an anti-p21 antibody. (D) NSΔRafER cells were

quiesced for 48 hours in DMEM supplemented with 2% FCS. The cells were then exposed to fresh medium supplemented with GGF, forskolin and 200nM TMX in the presence and absence of PD98059. The cells were lysed at the indicated times. The lysates were equalised for protein content and then western blotted using the anti-ERK-2 polyclonal antiserum 122 (top panel) or an anti-p21 antibody (bottom panel).

Figure 5. The kinetics of p21^{Cip1} induction, cyclin E-dependent kinase activity and DNA synthesis following Raf activation.

Asynchronously growing NSΔRafER cells were incubated in the presence of TMX (+) or control solvent (-).

(A)(o) Protein lysates were prepared at the times indicated. 300μg of the lysates were immunoprecipitated with an anti-cyclin E antibody and assayed for histone H1 kinase activity. (+) DNA synthesis was measured by the uptake of ³H-thymidine added between the times indicated. The results are expressed as the amount of activity relative to the corresponding control activity

(B) Protein lysates were prepared at the indicated time points or from quiescent NSΔRafER cells (Q) and were subjected to immunoblotting analysis with an anti-p21 antibody.

Figure 6. The induction of p21^{Cip1} in Raf-arrested cells is p53-dependent.

NSΔRafER cells were infected with retroviral vectors expressing a dominant-negative form of p53 (p53¹⁷⁵). Pools of NSΔRafERBp, NSΔRafERp53¹⁷⁵ and NSΔRafERLT cells were grown in the presence of TMX (+) or the control solvent (-) for 30 hours. (A)Protein lysates were prepared and analysed by western blotting with an anti-p21 antibody or an anti-cyclin D1 antibody as indicated.

- (B) 300µg of lysates were immunoprecipitated with an anti-cyclin E antibody and then analysed by western blotting with an anti-p21^{Cip1} antibody.
- (C) 300µg of lysates were immunoprecipitated with an anti-cyclin E antibody and assayed for histone H1 kinase activity
- (D) The NSΔRafERp53¹⁷⁵ cells were trypsinised and analysed for DNA content by propidium iodide staining and flow cytometry and for BrdU incorporation in the four hours prior to trypsinisation (see insets).

Figure 7. Raf and LT co-operate to induce high levels of cyclin A-dependent kinase activity and DNA synthesis.

(A) Left hand panel - Equal numbers of NSΔRafERBp, NSΔRafERp53¹⁷⁵, NSΔRafERLT cells were seeded into DMEM supplemented with 2% FCS. 48 hours later the cells were stimulated with fresh medium supplemented with 3% FCS, forskolin and GGF in the presence of TMX(+) or the control solvent(-). ³H-thymidine was added 12 hours later and the cells were harvested after a further 18 hours. TCA precipitable material was filtered and counted.

Right hand panel - NSΔRafERp53¹⁷⁵ cells were infected with the Babe-hygro retroviral vector constructed to express the cyclin A gene or the empty vector control to generate NSΔRafERp53¹⁷⁵cycA and NSΔRafERp53¹⁷⁵BH cells respectively. Hygromycin resistant colonies were pooled and expanded. DNA synthesis assays were performed as described above.

(B) Protein lysates were prepared from growing NSΔRafER, NSΔRafERp53¹⁷⁵ and NSΔRafERLT cells. 30µg of protein was analysed by western blotting with either an anti-cyclin A or anti-cdk2 antibody as indicated (upper two panels). 300µg of the lysates were immunoprecipitated with an anti-cyclin A antibody and assayed for histone H1 kinase activity (lower panel).

(C) NSΔRafERLT cells were seeded into DMEM supplemented with SATO mix (-mitogens) or DMEM supplemented with 3% FCS, GGF and forskolin (+mitogens) for 48 hours prior to the experiment. The cells were then treated with TMX (+) or control solvent (-) for 24 hours and protein lysates were prepared. 300μg of protein was immunoprecipitated with an anti-cyclin A antibody and kinase assays were performed with the precipitates. The kinase reactions were subjected to SDS-PAGE and then transferred onto a PVDF membrane. The filter was exposed to film (upper panel) and then western blotted with an anti-cdk2 antibody (lower panel).

Figure 8. Mechanisms of oncogene cooperation in Schwann cells (see also Conclusions).

Fig 1

A

NSARafter

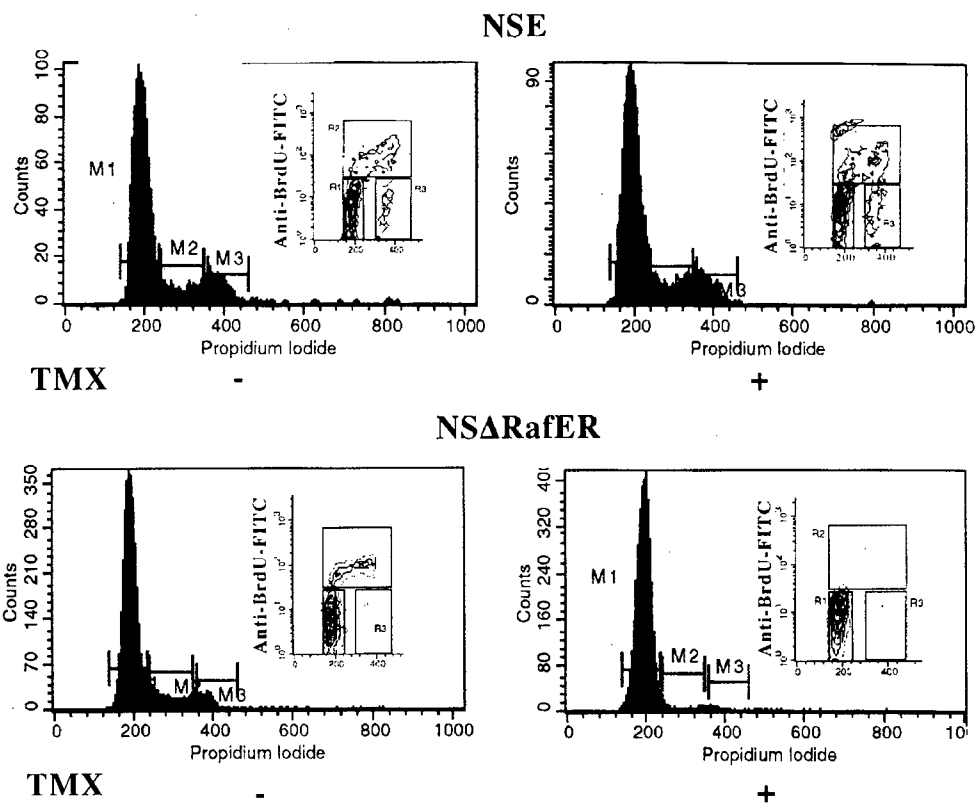


- TMX

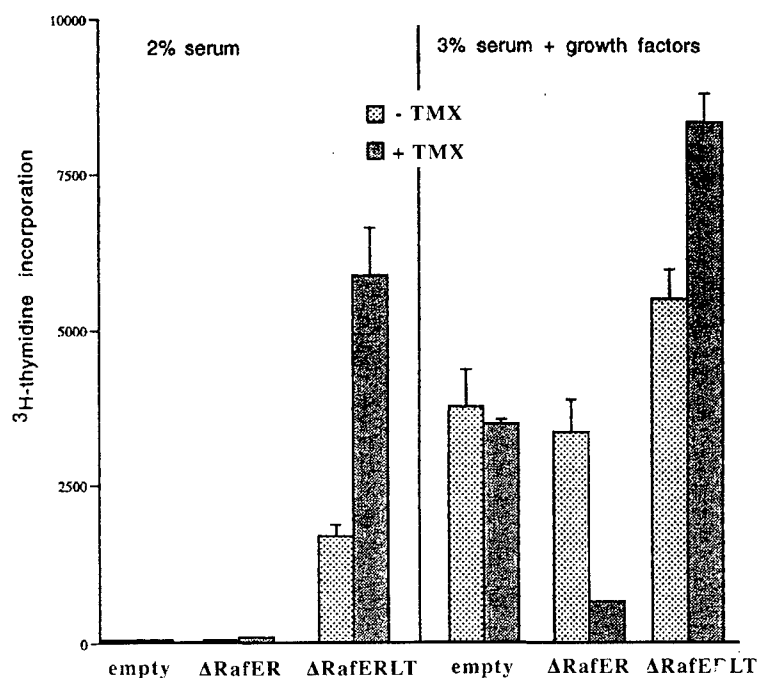
+ TMX

Fig 1

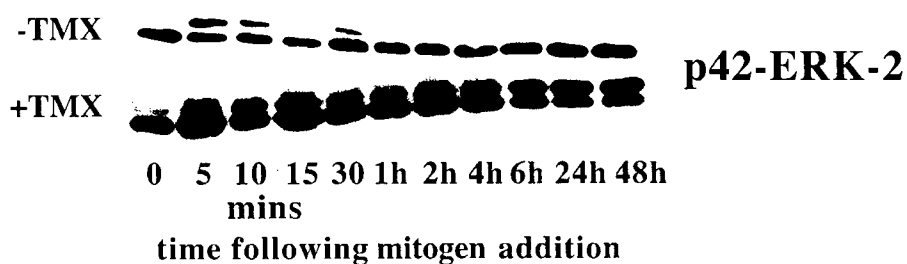
B



C



D



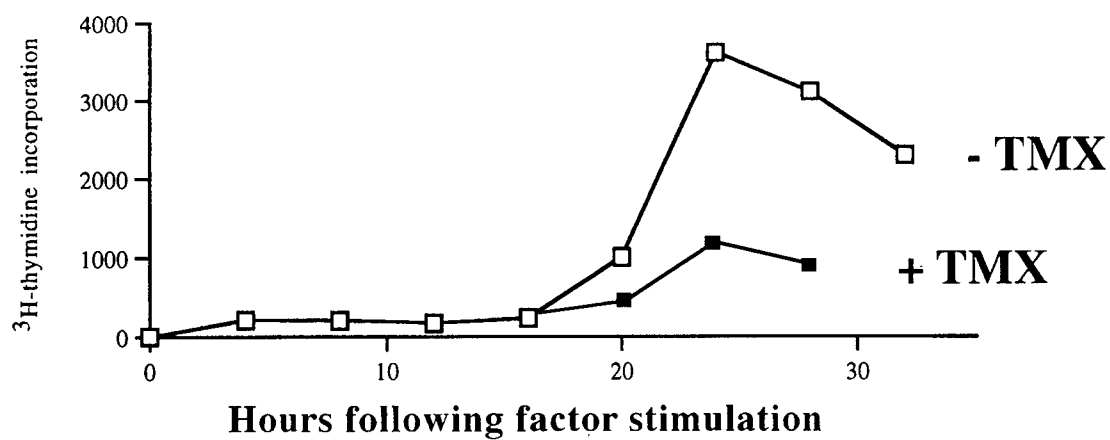
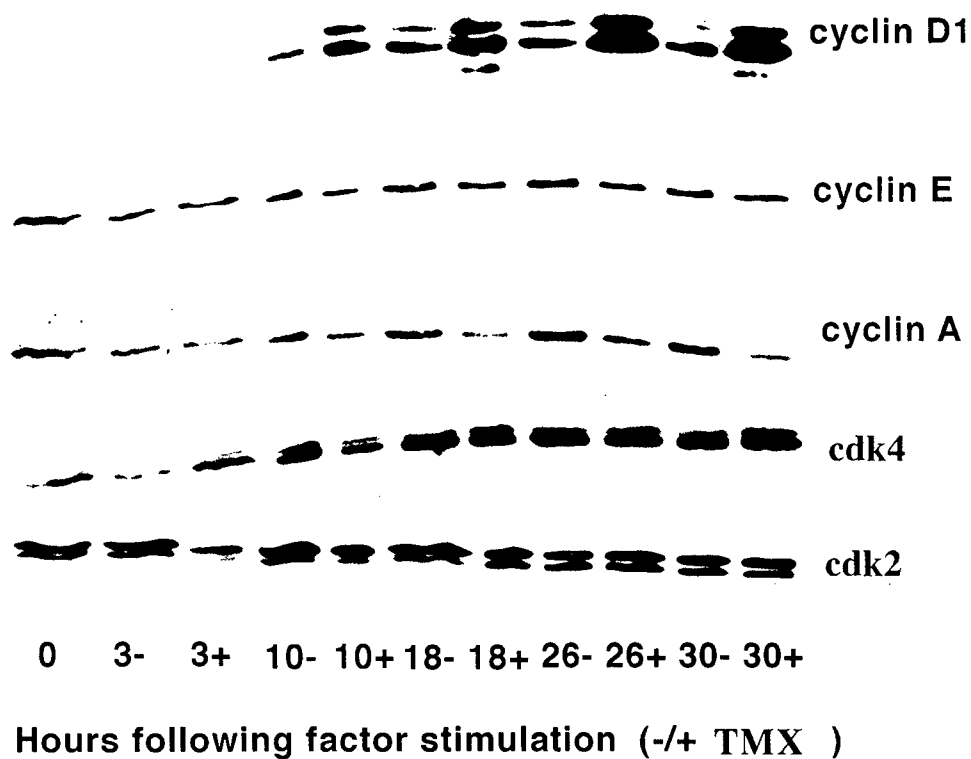
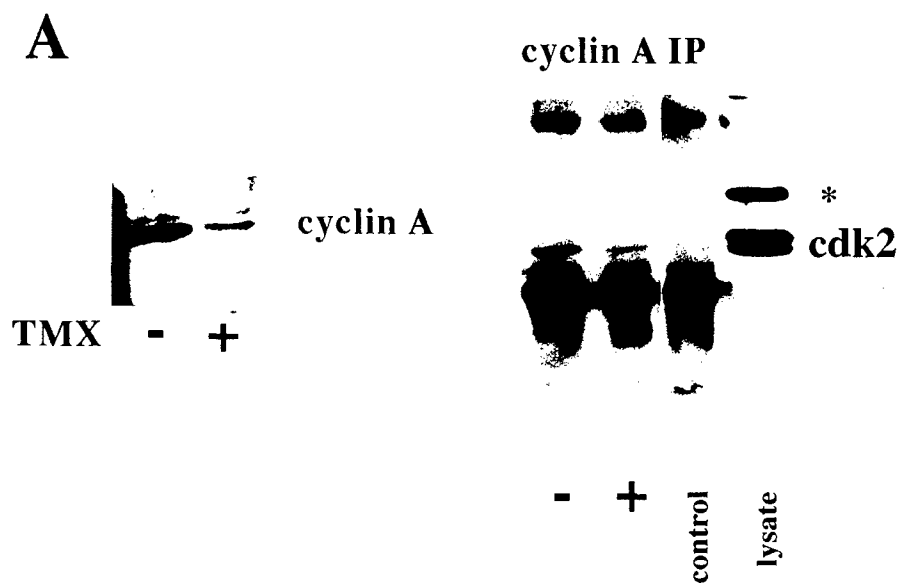
A**B**

Fig 3



cyclin A-dependent kinase activity

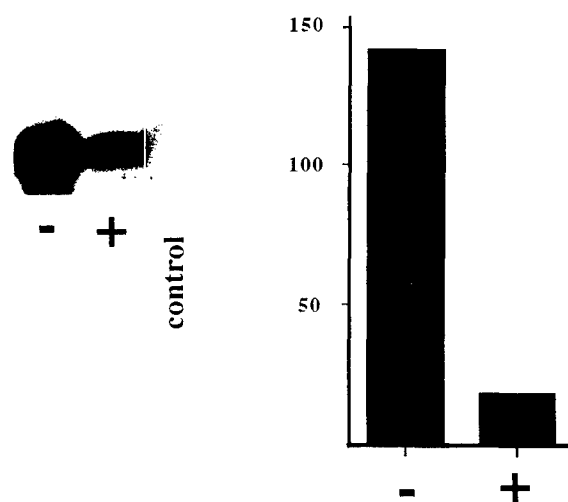
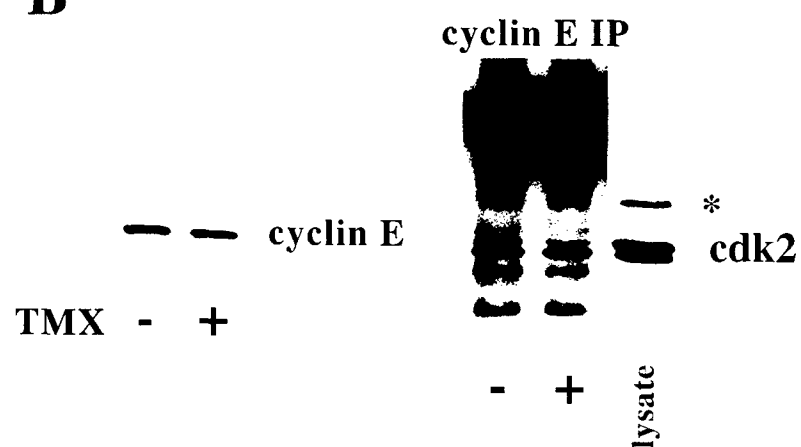


Fig 3

B



cyclin E-dependent kinase activity

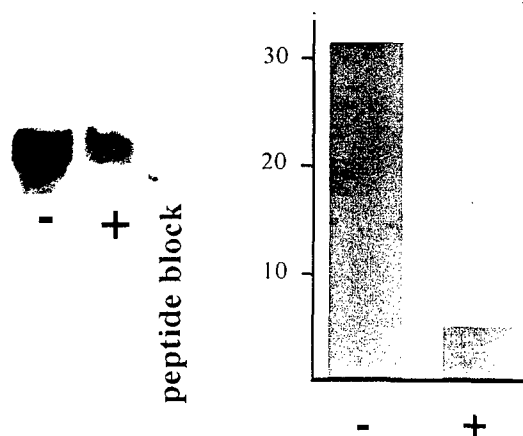


Fig 4

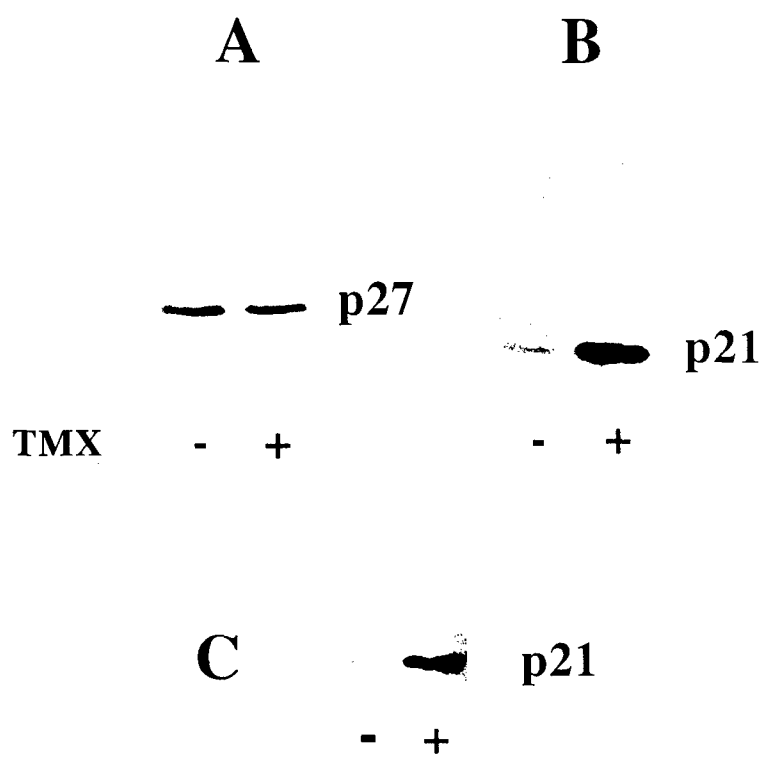


Fig 4D

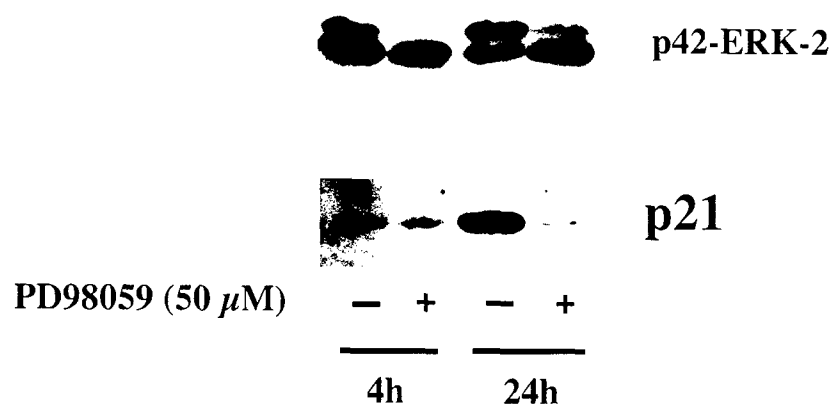
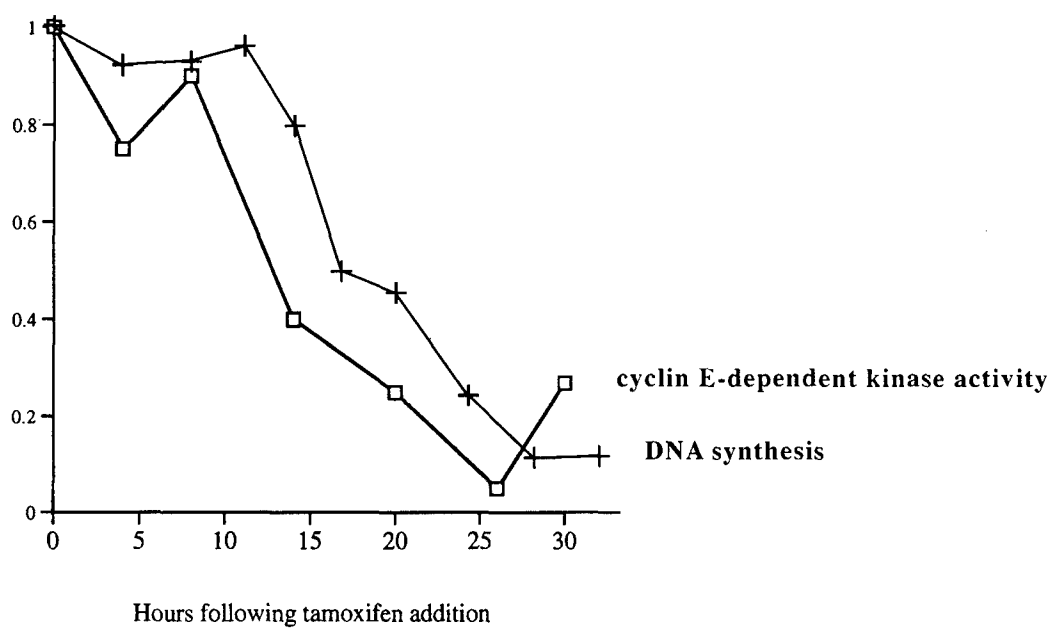


Fig 5

A



B

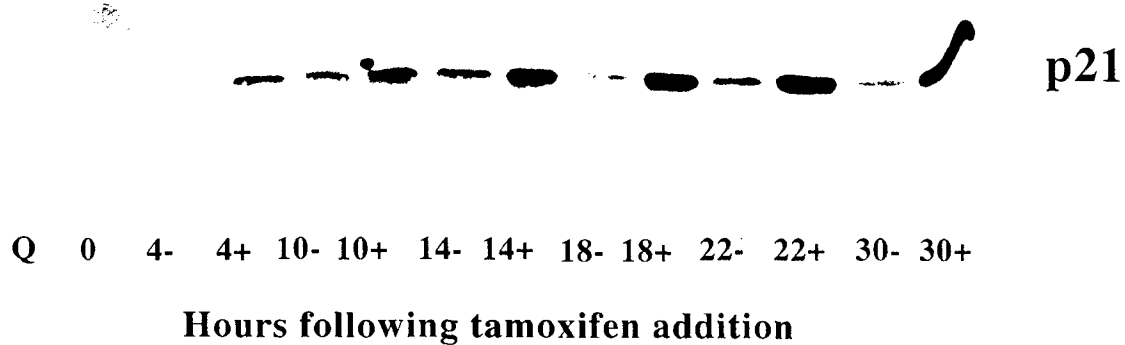


Fig 6

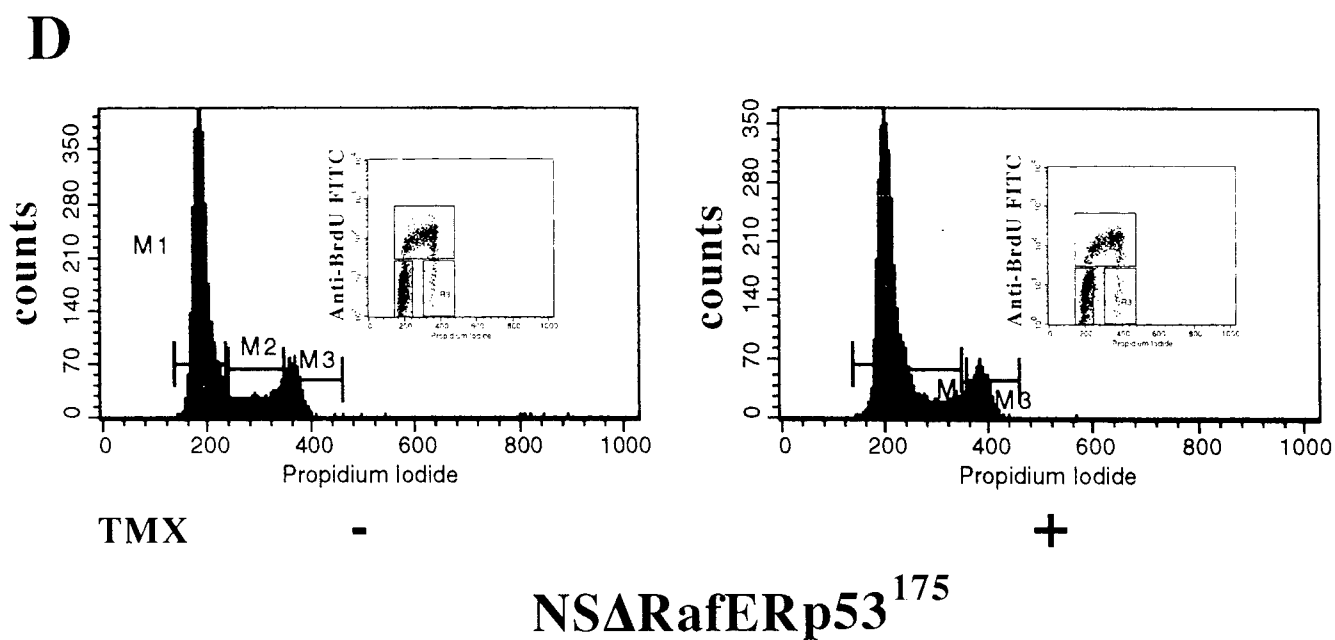
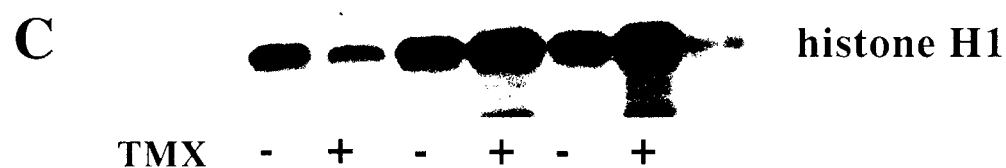
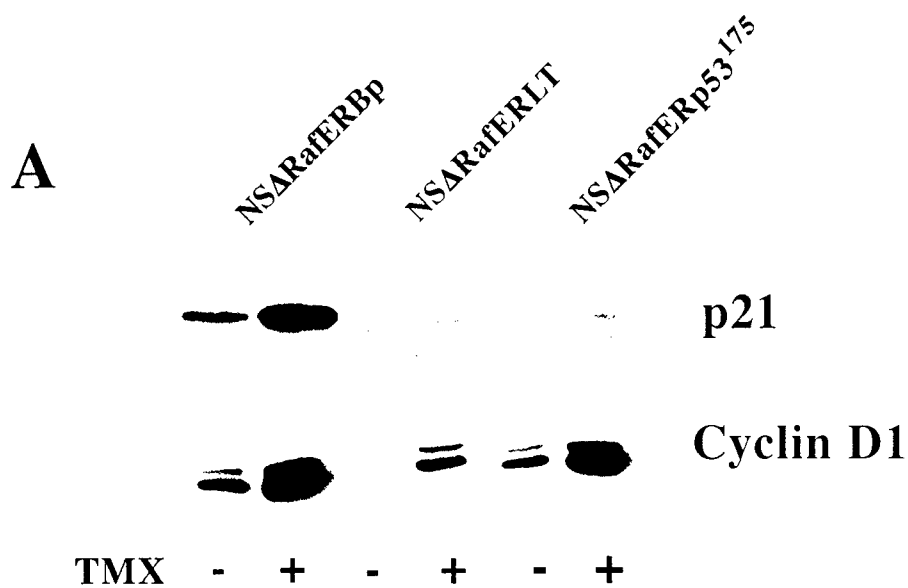
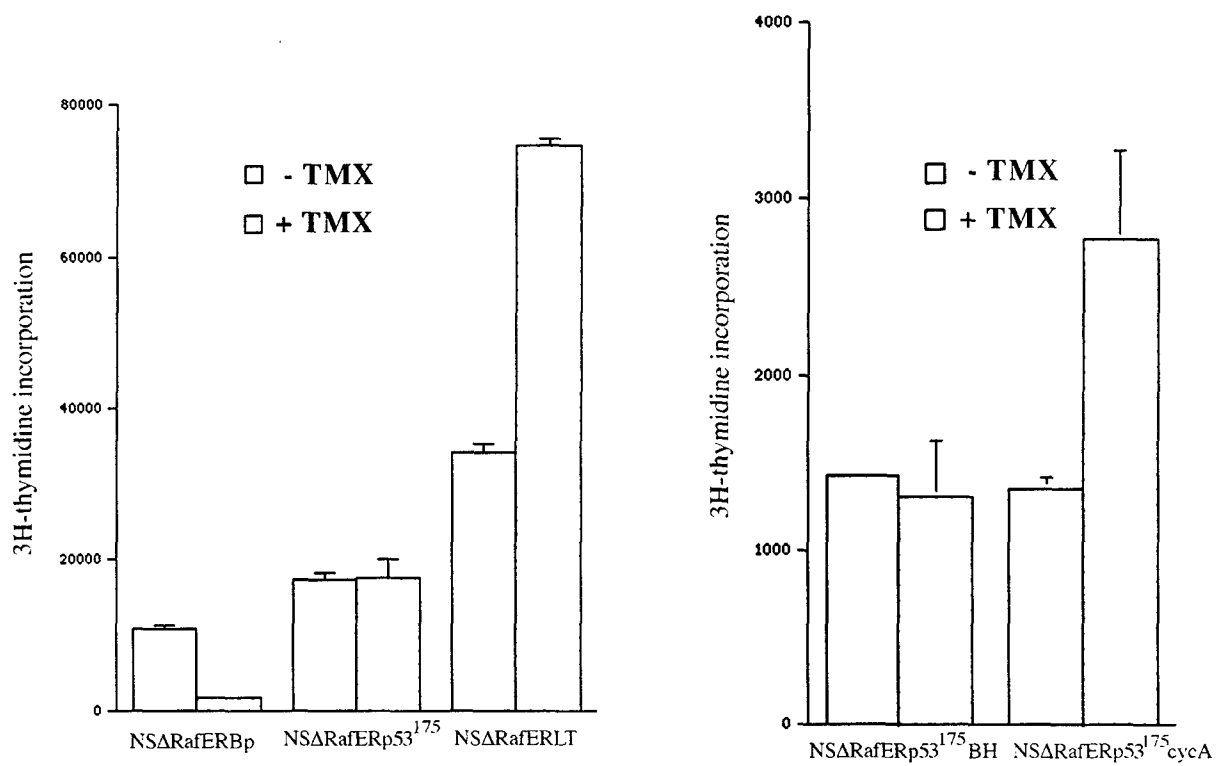
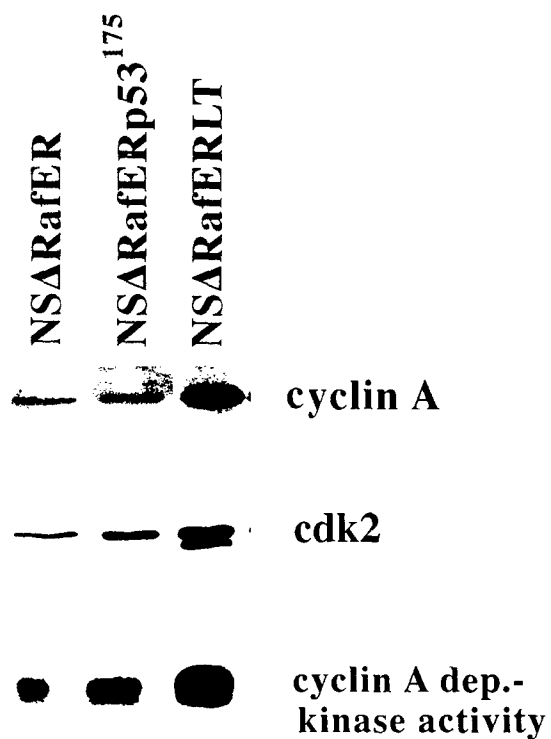


Fig 7

A



B



C

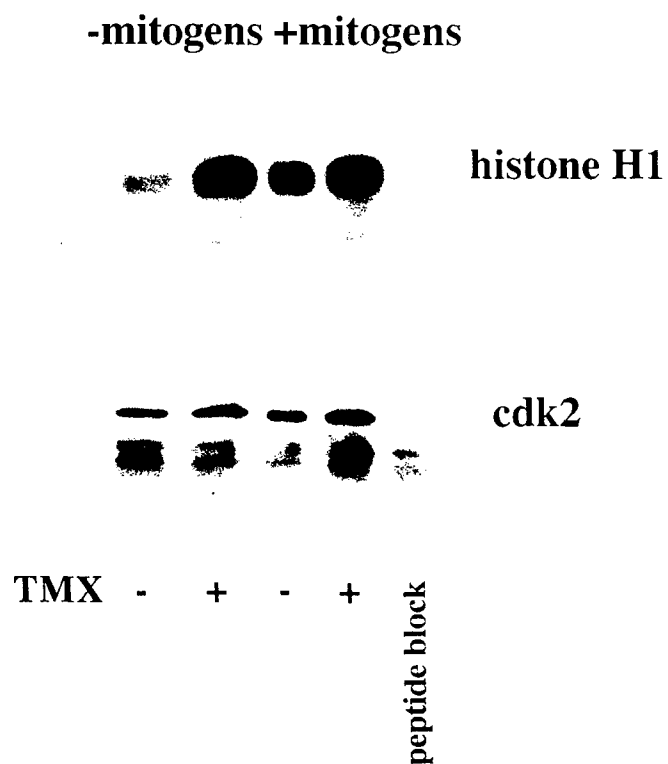


Fig.8

